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## GENOMICS AND HUMAN GENETICS

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### Old Dogs, New Tricks: Monogenic Autoinflammatory Disease Unleashed\*

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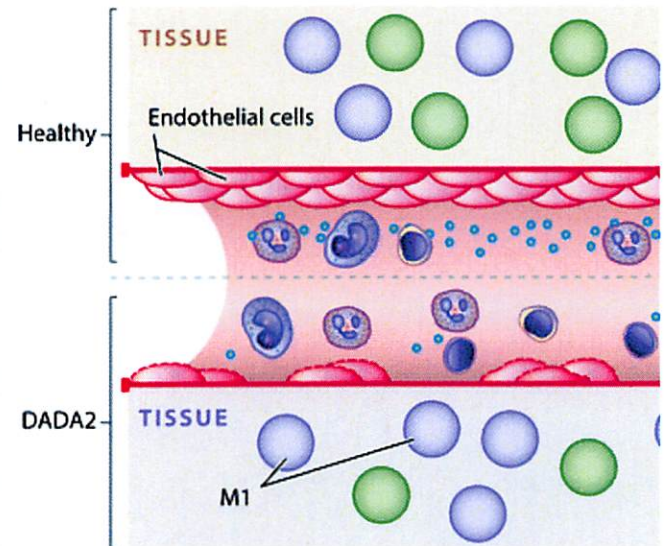
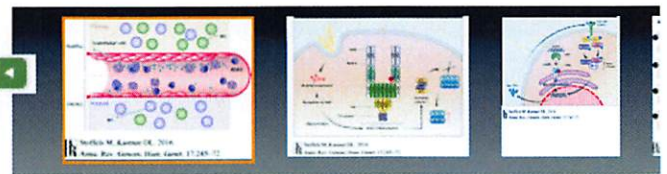
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Autoinflammatory diseases are inborn disorders of the system characterized by episodes of systemic inflammation mediated largely by myeloid cells. The field of autoinflammation has been established since 1999, following the identification of genes underlying periodic fever syndromes. This review discusses developments that have transformed the field in the last decade, including the discovery of new monogenic autoinflammatory diseases [deficiency of adenosine deaminase 2 (DADA2), a subset of macrophage activation syndrome (MAS), and stimulator of interferon genes (STING)-associated vasculopathy with onset in infancy (SIVA)], the possibilities of somatic mosaicism and digital medicine, and give an update on new concepts in pathways involved in the pathophysiology of several autoinflammatory diseases. Finally, the new monogenic disease haploinsufficiency of A20 (HA20) underscores the importance of monogenic diseases in the firmament of common autoimmune phenotypes. The advances in the last two years have elucidated new pathways that play a role in innate immunity.



Nature is nowhere accustomed more openly to her mysteries than in cases where she shows them apart from the beaten paths; nor is there any advance in the proper practice of medicine than in the discovery of the usual law of nature, by careful cases of rarer forms of disease.  
Sir William Harvey (1578–1657)

**AR** Stoffels M, Kastner DL. 2016.  
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FIGURE CAPTION

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The concept of systemic [autoinflammatory disease](#) was born upon the discovery of the genes underlying two distinct periodic fever syndromes: familial Mediterranean fever and Hibernian fever [subsequently renamed tumor necrosis factor receptor–associated periodic syndrome] (41, 60, 89). These manifest seemingly unprovoked, dramatic episodes of arthritis, and/or cutaneous inflammation, without the high autoantibodies or antigen-specific T cells usually associated with [autoimmune disease](#). With the discovery of the genes underlying four other rare periodic fever syndromes (6, 37, 39, 52, 55) and the recognition that additional monogenic disorders not usually thought to be periodic fever syndromes might nevertheless be considered autoinflammatory (92, 112), the concept gained currency.

**Figure 1** Vascular biology in DADA2: a working hypothesis as a growth factor for endothelial cells and M2 macrophages. ADA2 blood levels lead to impaired endothelial integrity and macrophages toward the more proinflammatory M1 phenotype, a vicious circle of vascular damage and inflammation. ADA2, deficiency of adenosine deaminase 2; DADA2, deficiency of adenosine deaminase 2.

The idea is now fully entrenched, owing largely to our emerging understanding of innate immunity and to the discoveries that linked autoinflammation to [innate immune system](#) pathology and autoimmunity to [adaptive immune system](#) pathology. The clinical science of autoinflammation and the basic science of innate immunity have developed *pari passu*, with the identification of new disease genes often illuminating previously unrecognized innate immune pathways, which have in turn led to spectacular therapeutic successes in the clinic. Such studies have provided the basis for a conceptual framework in which there is a spectrum of monogenic hyperimmune disorders, with the monogenic autoimmune diseases (such as autoimmune lymphoproliferative syndrome) at one end and the monogenic autoinflammatory diseases (such as FMF) at the other.



The early advances in this field were driven by the ascertainment of families segregating autoinflammatory phenotypes, studied under the lenses of linkage analysis, positional cloning, and genomic sequencing. This work has been thoroughly presented in several excellent reviews (29, 53, 85). Within approximately ten years, most of the clear-cut familial autoinflammatory diseases had been solved, leaving a large number of both early- and late-onset sporadic cases unexplained. With the recent advent of next-generation sequencing, there has been a renaissance in the field of autoinflammation, with the discovery of a host of new genes, pathways, and mechanisms of disease.

In this review, we chronicle some of the advances in the study of autoinflammation over the last two years. These include the discovery of several important new diseases, the recognition of somatic mosaicism in late-onset disease, and the demonstration of oligogenic inheritance in a disease of multiprotein complexes. We also highlight new insights into that oldest of autoinflammatory diseases, FMF, as genomics has met biology to transform our understanding of host-pathogen interactions. These recent breakthroughs in rare monogenic diseases have clearly enriched our understanding of the workings of innate immunity in general and, as such, also establish a firm foundation for investigating the genetic architecture of the more common, [complex autoinflammatory diseases](#).



Advances in genetics have increased awareness of the monogenic autoinflammatory diseases among a broad community of physicians and scientists. The increased rate of gene discovery has led to significant expansion of the autoinflammatory spectrum of diseases. Here, we focus only on the most recent advances, given the availability of comprehensive presentations of more established work (4, 5, 85, 114). Because currently fewer than half of patients with an autoinflammatory disease can be genetically diagnosed, new technologies promise to reveal many more genes that contribute to disease pathogenesis. It is often challenging to find these new genes; the autoinflammatory diseases appear to be more heterogeneous than previously thought, and many cases are not amenable to linkage analysis. Small sets of patients stimulate collaboration between clinical and basic researchers, and broad data sharing will be essential in this pursuit. Below, we discuss three recent discoveries that are interesting in their own right and have broad implications for both clinical medicine and basic science.

### 2.1. Deficiency of Adenosine Deaminase 2 and Vasculopathy

Early in 2014, two independent groups described autosomal recessive mutations in the *cat eye syndrome chromosome region, candidate 1* (*CECR1*) gene associated with both early-onset systemic inflammation and lacunar strokes and with polyarteritis nodosa (PAN) (94, 148). *CECR1* encodes the adenosine deaminase 2 (ADA2) protein (141), and hence the term deficiency of adenosine deaminase 2 (DADA2) has been proposed to denote patients with this genetic disorder. A cohort reported from the US National Institutes of Health (NIH) presented with recurrent fevers and livedo racemosa and had strokes involving the deep-brain nuclei and brain stem before the age of 5 (148). Other neurovascular manifestations, as well as hepatosplenomegaly and systemic vasculopathy, were also observed. Of particular note is that two brothers heterozygous for the p.Tyr453Cys *CECR1* mutation seen in DADA2 experienced late-onset strokes in the same anatomic distribution as DADA2 (148), suggesting a possible role for *CECR1* variants in complex forms of vascular disease.

A second cohort reported from Israel presented with PAN and included Georgian Jewish patients homozygous for a p.Gly47Arg founder mutation in *CECR1* (94). PAN is a systemic necrotizing vasculitis with a poorly understood pathogenesis. The most common features reported were livedo reticularis; gastrointestinal manifestations, including mesenteric arterial aneurysms and infarcts; renal artery stenosis with hypertension; and evidence of both peripheral and central nervous system involvement. Patients with severe disease had ischemia and necrosis of the fingers and toes.

The ADA2 protein is partially homologous to ADA, recessive loss-of-function mutations in which have long been recognized as a cause of severe combined immunodeficiency (125). Both proteins convert adenosine into inosine and 2'-deoxyadenosine to 2'-deoxyinosine, although the affinity of ADA is approximately 100 times higher than that of ADA2 (141). Moreover, ADA2 is dimeric and functions both intra- and extracellularly, whereas ADA functions intracellularly as a monomer. DADA2 patients have only a mild defect in B cell differentiation (148). In addition to its role as an enzyme, ADA2 may function as a growth factor in the ADA growth factor family (36, 59, 141, 142). Transiently knocking down the *cecr1b* paralog in developing zebrafish embryos resulted in intracranial bleeding and reduced numbers of neutrophils. Biopsy specimens from brain and lesional skin from DADA2 patients showed endothelial activation and damage (148). These data, taken together with the observations that (a) ADA2 is not ordinarily expressed in endothelial cells, (b) ADA2 levels are markedly reduced in the blood of DADA2 patients, and (c) ADA2 plays a preferential role in the differentiation of anti-inflammatory M2 macrophages, gave rise to the current working hypothesis for DADA2 pathogenesis (148) (Figure 1). According to this formulation, ADA2 deficiency prevents normal endothelial development, and the skewing of the resulting influx of macrophages toward the proinflammatory M1 subset leads to a vicious circle of endothelial damage and inflammation. Activated neutrophils may also contribute to this inflammatory process (10).

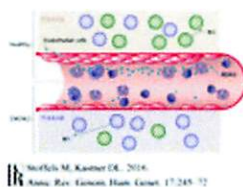


Figure 1

Experience from the Israeli cohort suggests a beneficial effect of TNF inhibitors (94) in DADA2-associated PAN, and preliminary analysis of data from the NIH supports a similar effect in patients presenting with stroke. More research into the pathogenesis may provide additional or complementary treatment options. Since the two initial reports on DADA2, multiple other papers have been

published (14, 43, 48, 68, 109, 128–130, 135). ADA2 mutations were found in patients with a phenotype similar to that of Castleman disease and could be treated with an interleukin 6 (IL-6) inhibitor (129), although others did not observe a role for IL-6 in their patients (68). Alternatively, hematopoietic stem cell transplantation has been tried in a small number of cases and resulted in resolution of the immunologic phenotype, correction of ADA2 blood levels, and no further strokes (128, 130).

As described above, ADA2 deficiency may unify a variety of syndromes previously thought to be distinct and may be important in other types of vasculitis or in other disorders that may present with stroke, such as Sneddon syndrome (14, 48, 94, 109, 148). It is important to think of DADA2 in young patients with unexplained lacunar strokes even in the absence of a positive family history (135).

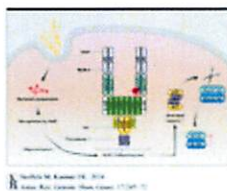
## 2.2. NLRC4 Inflammasome Activation in Early-Onset Fever and Macrophage Activation Syndrome

Inflammasomes are complex protein structures formed upon recognition



of pathogen- or damage-associated signals by innate immune receptors on the cell membrane and in the cytoplasm. Stimulation of these receptors leads to activation of caspase-1 by the inflammasomes, pyroptosis, and secretion of the proinflammatory cytokines IL-1 $\beta$  and IL-18 (83, 118). Gain-of-function dominant mutations in *NOD-like receptor (NLR) family, pyrin domain containing 3 (NLRP3)*, encoding a component of the inflammasome of the same name, have been shown to cause constitutive caspase-1 activation and the [autoinflammatory disease](#) cryopyrin-associated periodic syndrome (CAPS) (6, 39, 52). CAPS patients almost always show a dramatic response to therapies that block IL-1 $\beta$ , demonstrating the central role of this cytokine in driving the phenotype (46). By extension, this response has provided the conceptual basis for often successful application of IL-1-blocking therapies in other autoinflammatory diseases (23, 34).

In 2014, two groups independently reported an autoinflammatory disorder associated with a second member of the NLR gene family called *NLR family, caspase recruitment domain (CARD) containing 4 (NLRC4)* (22, 104). The NLRC4 protein cooperates with NLR family, apoptosis inhibitory protein (NAIP) to detect cytosolic flagellin and components of the type 3 secretion system used by some bacteria to infect hosts (90, 121). Upon ligand binding, NLRC4 and NAIP oligomerize and recruit apoptosis-associated speck-like protein with a CARD (ASC), leading to the aforementioned activation of caspase-1, secretion of IL-1 $\beta$  and IL-18, and pyroptosis (17, 84, 91, 121) (**Figure 2**).



**Figure 2**

Romberg et al. (104) described a family in which the index case presented in the first week of life with secretory diarrhea and fever accompanied by elevated serum levels of systemic inflammatory markers, including hyperferritinemia. This patient died after 23 days of life from diffuse alveolar hemorrhage. The father had a lifelong history of recurrent fevers induced by physical and emotional

stressors. His other child, a half brother of the deceased infant, also had recurrent fevers beginning on day 3 of life. Canna et al. (22) described a patient with recurrent episodes of fever, malaise, splenomegaly, vomiting, loose stools with mild duodenitis, and occasional rash beginning at 6 months of age. The combination of elevated inflammatory markers, chronic anemia, elevated transaminases, hypertriglyceridemia, hyperferritinemia, leukopenia, and thrombocytopenia with severe flares was much more consistent with macrophage activation syndrome (MAS) than with CAPS.

MAS and the related hemophagocytic lymphohistiocytosis (HLH) are life-threatening systemic immune system dysregulatory conditions (21, 149). MAS is sometimes seen in association with certain rheumatic diseases (54, 101), and both MAS and HLH can be triggered by infections and malignancies. Based on mutations found in genes necessary for perforin- and granzyme-mediated killing in familial HLH, it is hypothesized that impaired cytotoxic cell killing leads to uncontrolled activation and survival of macrophages and dendritic cells (98, 124, 149). However, Romberg et al. (104) and Canna et al. (22) found evidence of a macrophage-intrinsic abnormality that can drive the MAS phenotype in the absence of a primary cytotoxic defect.

Whole-exome sequencing revealed two independent mutations in the highly conserved nucleotide-binding domain of NLRC4, p.Thr337Ser and p.Val341Ala, which are predicted to cause impaired autoinhibition of NLRC4 (**Figure 2**). Consistent with findings in MAS in systemic juvenile



idiopathic arthritis, adult-onset Still's disease, infection, and X-linked inhibitor of apoptosis (XIAP) deficiency (58, 87, 95, 111, 133), the patients exhibited elevated levels of serum IL-18 that were higher than those in CAPS patients. Both Romberg et al. (104) and Canna et al. (22) used transfection studies and stimulation of peripheral blood monocytes and macrophages to study the functional consequences of the mutations. The NLRC4 mutations led to higher spontaneous ASC-dependent caspase-1 cleavage, pyroptosis, and more IL-1 $\beta$  and IL-18 secretion. Canna et al. (22) also saw high levels of IL-1 $\beta$  secretion in patients with neonatal-onset multisystem inflammatory disease (NOMID), also known as chronic infantile neurologic cutaneous articular (CINCA) syndrome. However, the spontaneous secretion of IL-18 was specific for the NLRC4-MAS monocytes and macrophages. These results suggest that the NLRC4 mutations lead to a gain of function through constitutive caspase-1 activation, and point to a differential role for the NLRP3 and NLRC4 inflammasomes in inducing IL-18 production and secretion. NLRC4 is different from NLRP3 in its association with neonatal-onset enterocolitis, which Romberg et al. (104) suggested may be due to the high expression of NLRC4, compared with NLRP3, in intestinal macrophages (40).

Given its molecular pathogenesis, this condition has been denoted the syndrome of enterocolitis and autoinflammation associated with mutation in *NLRC4* (SCAN4), or NLRC4-MAS. Canna et al. (22) were able to offer their patient IL-1-blocking treatment (anakinra), which is effective in several autoinflammatory diseases (23, 34) and resulted in a marked decrease in fevers and clinical flares. However, subclinical inflammation persisted. The continuance of high IL-18 serum levels after IL-1 inhibition warrants further exploration of IL-18-targeted therapies.

The results from these studies not only explain the pathogenesis of a few rare patients with autoinflammatory findings, but also reveal yet another [innate immune](#) pathway that is implicated in autoinflammation. Furthermore, the patients described in these studies fit the criteria of MAS, a complication of other autoinflammatory and [autoimmune diseases](#), and patients with adult-onset Still's disease are known to have high IL-18 serum levels (100). Discovering the pathogenesis in SCAN4/NLRC4-MAS may hold promise for elucidating the pathobiology in other patients with MAS and warrant further exploration of IL-18-targeted therapies, such as the IL-18-binding protein or monoclonal antibodies against IL-18. Interestingly, Kitamura et al. (71) found a mutation in *NLRC4* that causes a phenotype that much more resembles familial cold autoinflammatory syndrome, a much milder phenotype than MAS. This could perhaps be explained by the location of the *NLRC4* mutation.

### 2.3. STING-Associated Vasculopathy with Onset in Infancy

The discovery of stimulator of interferon genes (STING)–associated vasculopathy with onset in infancy (SAVI) represents a paradigm-shifting convergence of clinical genetics and basic immunology, in much the same way that the nearly contemporaneous elucidation of CAPS and the NLRP3 inflammasome transformed the field of autoinflammation a decade earlier. The discovery of a dramatic clinical phenotype caused by gain-of-function de novo mutations (78) in a novel cytosolic DNA sensor (119, 137) underscored the importance of that sensor in the innate immune system and has solidified the concept of type I interferon (IFN)–mediated autoinflammatory disease.

The story begins with the 2008 discovery of the STING protein, encoded by *transmembrane protein 173* (*TMEM173*) (61, 65, 120, 145). STING is essential for IFN- $\beta$  induction and plays a role in responses to viral and bacterial pathogens and to self-DNA. Its action can have a protective role,

as in the recognition of tumor cell DNA (33, 72, 136), but it can also play a devastating role in driving inflammatory diseases that are caused by the accumulation of self-DNA (3). Chen and colleagues (42, 75, 119) recently identified the key elements in this pathway, demonstrating in an elegant series of *Science* papers that the cytosolic DNA sensor cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) synthase (cGAS) catalyzes cGAMP synthesis in response to intracellular double-stranded DNA, that cGAMP is an endogenous second messenger that binds to STING in the endoplasmic reticulum and leads to the activation of IFN regulatory transcription factor 3 (IRF-3) and the induction of IFN- $\beta$  (1, 77, 137) and other cytokines (19), and that this pathway is the primary trigger for production of type I IFNs in response to viral infections (42, 75, 119) (Figure 3). Although the induction of the IFN response is essential for detection and clearance of viral pathogens (115), cGAS also detects *Mycobacterium tuberculosis* (27), triggering the activation of IFN genes important for cytokine production and induction of autophagy.

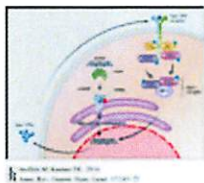


Figure 3

The following year, Goldbach-Mansky and colleagues (78) demonstrated that gain-of-function mutations in STING define a new human disease: SAVI. This study described six patients who experienced symptoms before 8 weeks of age and whose illnesses included systemic inflammation, cutaneous rash, pulmonary manifestations such as interstitial lung disease, and severe small vessel vasculopathy. All had failure to thrive, and two died of pulmonary complications and secondary infection. Using

exome and Sanger sequencing, the authors identified mutations in *TMEM173* as the likely cause. The mutations lead to the constitutive activation of the STING–IFN- $\beta$  pathway. In agreement with this, the group found a strong transcriptional IFN-response-gene signature and elevated levels of IFN-inducible protein 10 (IP-10) and other IFN-induced cytokines in the peripheral whole blood of patients. By in vitro experiments, they also found that Janus kinase (JAK) inhibitors suppress the expression of STING-induced targets and IFN-response genes in patients' lymphocytes, and a therapeutic trial of JAK inhibition in SAVI is currently under way. The recognition of SAVI underscores the importance of the cGAS–cGAMP–STING–IFN pathway in innate immunity and suggests STING as a potential therapeutic target not only in rare autoinflammatory conditions such as SAVI, but also in other, more common double-stranded DNA–driven interferonopathies, including systemic lupus erythematosus.



### 3.1. Mosaicism

Above, we discussed only monogenic diseases caused by germline mutations, meaning that all cells in the body contain DNA with that given mutation. However, sometimes a disease results from the presence of mutations only in certain populations of cells that were derived from the same fertilized egg as the rest of the cells in the organism, a phenomenon called mosaicism (as distinguished from chimerism, in which independent lineages derive from more than one fertilized egg). Mosaic mutations may be acquired over time, explaining the possibility of late-onset disease. A well-known example is cancer, in which multiple mutations in tumor suppressor genes are sometimes acquired in a cell lineage over many years. Mosaicism can also happen early during



embryogenesis; an individual then carries a mutation in all cells descended from that one cell during embryonic development. Depending on how early or late in embryogenesis this happens, a relatively large or small number of cells in the adult carry that mutation. Although postzygotic mosaicism is a well-recognized cause of cancer, it has been harder to document in nonmalignant conditions, where the relevant cell type and gene may be unknown (11).

Detection of mosaicism by standard Sanger sequencing is often difficult. The low percentage of DNA strands carrying the mutation causes at best a small bump in the electropherogram and usually cannot be distinguished from background noise (Figure 4a). Massively parallel sequencing techniques such as whole-exome sequencing and targeted deep resequencing provide a possible solution to this problem.

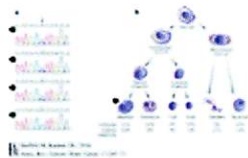


Figure 4

### 3.2. The Cryopyrin-Associated Periodic Syndrome Spectrum

CAPS is a spectrum of rare autosomal dominant [autoinflammatory diseases](#) caused by mutations in the *NLRP3* gene (6, 39, 52).

*NLRP3* encodes an intracellular pattern recognition receptor that forms the *NLRP3* [inflammasome](#), which is responsible for

processing pro-IL-1 $\beta$  and pro-IL-18 to the biologically active IL-1 $\beta$  and IL-18, respectively, two major proinflammatory cytokines. The different forms of CAPS share recurrent fevers, joint pain, and neutrophilic urticaria-like rashes, but each form also has its own distinctive features (reviewed in 85). The mildest form is familial cold autoinflammatory syndrome, symptoms of which can be induced by cold exposure. Muckle-Wells syndrome (MWS), the intermediate form, also includes sensorineural hearing impairment and secondary amyloidosis in 25% of patients. The most severe phenotype is NOMID/CINCA, which arises mainly from de novo mutations, with no family history. Patients with this neonatal-onset form of CAPS suffer from arthropathy; urticaria; bone and joint deformities (most characteristically overgrowth of the epiphyses of the long bones); chronic aseptic meningitis leading to intellectual disability, blindness, and deafness; and systemic amyloidosis.

In general, CAPS patients respond well to IL-1 inhibition. Therefore, correctly diagnosing and establishing disease pathology is important. Approximately 40% of the clinically diagnosed NOMID/CINCA patients are mutation negative, as established by standard Sanger sequencing.

### 3.3. *NLRP3* Mosaicism in Cryopyrin-Associated Periodic Syndrome

A significant number of mutation-negative CAPS patients can be explained by *NLRP3* mosaicism, which also accounts for the apparent de novo transmission in some cases. By Sanger sequencing more than 50 subcloned polymerase chain reaction amplicons from exon 3 of *NLRP3*, Saito et al. (105) were the first to report low-level *NLRP3* mosaicism as the cause of a sporadic case of NOMID/CINCA, in 2005. They found that the frequency of the mutant allele in whole blood was 16.7%, corresponding to approximately 33% of whole-blood cells being heterozygous for the *NLRP3* mutation. This study was important because it provided an explanation for at least some cases of mutation-negative CAPS. Because subcloning is laborious and impractical for diagnostic purposes, the group went on to attempt the enrichment of *NLRP3*-mutated cells, which would enhance the probability of finding relevant mutations. To this end, they found that monocytes carrying an *NLRP3* mutation rapidly undergo necrosis-like death after lipopolysaccharide exposure; by purifying such dying cells, they found additional cases of low-level mosaicism in *NLRP3* in four CAPS patients (one with MWS and



three with NOMID/CINCA) (106).

In an international multicenter collaborative study, Tanaka et al. (123) investigated a panel of 26 mutation-negative NOMID/CINCA patients in order to determine how many actually had *NLRP3* mosaicism. Sanger sequencing 96 subcloned *NLRP3* exon 3 amplicons for each patient showed that 18 of the 26 patients (69.2%) had *NLRP3* mosaicism. The degree of allelic mosaicism ranged from 4.2% to 35.8% in whole blood. All of the variants they identified were pathogenic, as established by cell death assays and nuclear factor kappa B (NF- $\kappa$ B) activation assays in THP-1 cells. They could not detect a significant difference in mutation frequency among neutrophils, monocytes, T cells, and B cells. These data suggest that, in addition to the 60% of NOMID/CINCA patients with *NLRP3* germline mutations, 28% more may harbor mosaicism. The remaining 12% may be true mutation-negative cases, although it cannot be excluded that these patients carry pathogenic variants in the 5' untranslated region or are extremely low-level mosaics, as the detection limit for mosaicism in this study was 5%.

Massively parallel sequencing using next-generation sequencing platforms can increase the speed and sensitivity of screening for mosaicism while reducing the labor involved. One approach is to utilize a targeted deep resequencing strategy in which *NLRP3* amplicons are sequenced to a read depth of several hundred reads. Using such an approach, Izawa et al. (62) were able to detect somatic mutants with allele frequencies as low as 1% with >99% confidence at a read depth of ~350x. *NLRP3* mosaicism has also been identified by whole-exome sequencing in NOMID/CINCA (97), but the success of this approach is highly dependent on the capture kit, average read depth, and degree and tissue distribution of mosaicism in the patient. In the cited example, the mutant allele frequency was 17.7% in whole blood by whole-exome analysis and 14.5% by targeted deep resequencing. Although low-level mosaicism is opaque to whole-exome sequencing at typical read depths, this strategy does have the advantage of interrogating the rest of the genome for both germline and mosaic mutations, at a modest level of sensitivity.

*NLRP3* mosaicism is not restricted to NOMID/CINCA patients. Using targeted next-generation deep resequencing, Nakagawa et al. (93) recently found mosaicism in 7 MWS patients from a panel of 56 mutation-negative CAPS patients, none of whom had NOMID/CINCA. Mutant allele frequencies ranged from 5.5% to 34.9%. From a comparison of 41 MWS patients harboring germline *NLRP3* mutations with the 7 mosaic patients, it appeared that patients with somatic mutations have a later onset of disease and sensorineural symptoms, an increased coincidence of arthritis, and a reduced risk of AA amyloidosis.

The possibility of mosaicism has important implications for genetic counseling. In one reported case, what was initially thought to be a de novo case of MWS proved instead to be vertical transmission from a clinically healthy mother harboring an *NLRP3* mutation at an allele frequency of 2.4–8.2% in the various tissues assayed (64). Asymptomatic parental mosaicism thus becomes an important consideration in CAPS cases thought to be de novo. This case also underscores the limitations in our understanding of the determinants of penetrance for mosaic *NLRP3* mutations, which are likely to include the specific mutation, its allele frequency, and the tissue distribution.

Although the case for mosaicism in typical early-onset CAPS has been building for the last decade, over the last year it has become clear that mosaicism also accounts for at least some cases of later-onset disease (31, 146). De Koning et al. (31) studied a cohort of patients with

Schnitzler syndrome, a rare late-onset autoinflammatory disease with a slight male predominance that, on clinical grounds, resembles CAPS (76, 108). The main manifestations are a neutrophilic urticaria-like skin rash and the production of monoclonal immunoglobulin M (IgM) antibodies, and other findings include lymphadenopathy, hepatosplenomegaly, arthralgia, and myalgia. Schnitzler syndrome patients may also develop an inflammatory anemia and renal amyloidosis, and 20% can develop a lymphoproliferative disorder (76). IL-1 blockade is very effective in these patients (30, 113). Using targeted deep resequencing of the *NLRP3* gene, de Koning et al. (31) identified 2 out of 11 patients as *NLRP3* mosaics. Notably, these two patients were the most severely affected in the cohort and had an IgG rather than an IgM gammopathy. Mosaicism was restricted to the myeloid cell lineage; mutations were present in granulocytes and monocytes but not in T cells, B cells, or keratinocytes. Peripheral blood mononuclear cells from these two patients also constitutively produced IL-1 and IL-6, confirming the pathogenicity of the mutations. The authors suggested that Schnitzler syndrome patients with *NLRP3* mosaicism should be added to the CAPS spectrum (31). They also noted that if disease severity is linked to the degree of mosaicism, they cannot exclude extremely low-level mosaicism (below the current detection limit) in the other patients.

Zhou et al. (146) subsequently described a middle-aged patient with a recent-onset illness resembling MWS who lacked the monoclonal gammopathy and bone pain associated with Schnitzler syndrome. Using whole-exome sequencing and targeted deep resequencing, they found that this patient harbored *NLRP3* mosaicism with an allele frequency of 10.9%. Subcloning and targeted deep resequencing showed that *NLRP3* mutations were present in monocytes and granulocytes (allele frequencies of 13.3–16.8% and 15.2–18%, respectively) but not in T cells, fibroblasts, or buccal cells (Figure 4b). Together, the papers described above indicate that mosaicism plays a role in late-onset CAPS and CAPS-like disorders, with mutations restricted to the myeloid lineage.

With faster and more efficient sequencing techniques and bioinformatics and advances in our understanding of basic immunology, it is likely that the example of *NLRP3* mosaicism will serve as a model for both early- and late-onset autoinflammatory disease, accounting for an increasing percentage of heretofore unexplained cases. Whether mosaicism for mutations at [innate immune](#) loci will fall into the spectrum of malignancy will depend on whether those mutations confer some growth advantage. In the case of CAPS-associated gain-of-function *NLRP3* mutations, there is an increased proclivity to pyroptosis and cell death, and thus a natural check against malignant transformation. By contrast, the gain-of-function p.Leu265Pro mutation in the innate signaling molecule MYD88 strongly associates with Waldenström macroglobulinemia (126), perhaps vindicating a possible linkage between autoinflammation and malignancy through somatic mosaicism.



#### 4.1. Digenic Inheritance

Whereas monogenic traits are caused by one or two copies of mutations in a single gene, following Mendelian inheritance patterns, many other inherited diseases reflect a complex interaction of multiple genes with environmental risk factors. At an intermediate place on the continuum are the [digenic](#) diseases—the simplest form of complex inheritance, but nevertheless relatively difficult to document (107, 132). In cases where there seems to be monogenic inheritance with reduced penetrance, a



two-locus model may provide an explanation (107, 132). To manifest as a digenic trait, at least one allele at each of the two loci needs to contain a mutation. Those loci usually generate products with synergistic functions. Examples include mutations in different subunits of a multimeric protein and mutations in genes that are in the same biologic pathway, compromising the overall function of that pathway. There are relatively few well-documented digenic disorders (107, 132), which include some cases of retinitis pigmentosa (67), digenic nonsyndromic deafness (32), and Usher's syndrome (144). An example of a digenic disease with triallelic inheritance is Bardet-Biedl syndrome, a Mendelian recessive disorder (69). In 2015, Brehm et al. (16) described the first autoinflammatory disorder with digenic inheritance.

#### 4.2. Digenic Inheritance in Proteasome-Associated Autoinflammatory Syndrome

Autosomal recessive homozygous or compound heterozygous mutations in the *proteasome subunit beta type 8* (*PSMB8*) gene cause disorders that belong to the proteasome-associated autoinflammatory syndrome (PRAAS) spectrum, variously described as the syndrome of joint contractures, muscle atrophy, microcytic anemia, and panniculitis-induced lipodystrophy; Nakajo-Nishimura syndrome; Japanese autoinflammatory syndrome with lipodystrophy; and chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE) (2, 8, 70, 79, 88). Among the most prominent clinical features are early-onset recurrent fever, nodular skin rash, myositis, panniculitis-induced lipodystrophy, and basal ganglion calcifications. Proteasomes break down intracellular proteins that are labeled for degradation through ubiquitination. The proteasome is a barrel-shaped structure consisting of a catalytic core (20S proteasome) and two regulatory 19S subunits (49, 56, 110) (Figure 5). Briefly, the core consists of two pairs of heptameric rings, containing  $\alpha$  and  $\beta$  subunits. In the constitutive proteasome expressed in all cell types, the three proteolytic active sites in each  $\beta$  ring are located in subunits  $\beta 1$ ,  $\beta 2$ , and  $\beta 5$ , each of which is preferentially inclined to cleave after an acidic, basic, or hydrophobic amino acid, respectively. The  $\beta 5$  subunit is involved in generating epitopes that are bound to major histocompatibility complex class I (MHC I). Although the constitutive proteasome is most predominant in steady-state conditions, exposure to IFNs favors the incorporation of the so-called inducible subunits:  $\beta 1i$ ,  $\beta 2i$ , and  $\beta 5i$ . A proteasome of this composition is called an immunoproteasome and is constitutively expressed in antigen-presenting cells, generating more MHC I peptides with hydrophobic or basic C termini. The  $\beta 1i$ ,  $\beta 2i$ , and  $\beta 5i$  subunits have caspase-like, trypsin-like, and chymotrypsin-like activities, respectively.

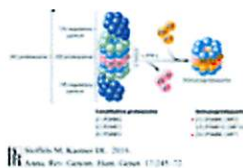


Figure 5

Mutations in *PSMB8* lead to a defect in  $\beta 5i$  incorporation into the immunoproteasome that impairs its chymotrypsin activity. The result is an accumulation of ubiquitinated and oxidized proteins in the cell. In CANDLE syndrome, part of the PRAAS spectrum, two mutations in *PSMB8*/ $\beta 5i$  cause a reduction of proteasome activity that leads to a unique IFN signaling gene expression profile and very high blood

levels of IP-10 (79). Increased type I IFN production leads to the production of reactive oxygen species and newly synthesized proteins that are particularly sensitive to oxidation, and the accumulation of such damaged proteins in the face of defective proteasome function can thereby lead to a vicious circle of increased IFN signaling (15, 79).

In contrast to previous reports describing monogenic *PSMB8* mutations in PRAAS, Brehm et al. (16) demonstrated digenic inheritance of *PSMB8*

with additional proteasome subunits that were previously not associated with disease. The authors included eight CANDLE patients in their study who had either no mutation or only one mutation in the *PSMB8* gene. They found one patient carrying compound heterozygous mutations in *PSMB4*/β7, two patients with one *PSMB8* and one *PSMA3*/α7 mutation, two patients with one *PSMB4*/β7 and one *PSMB9*/β1i mutation, two siblings with one *PSMB4*/β7 and one *PSMB8*/β5i mutation, and one patient heterozygous for a frameshift mutation in *proteasome maturation protein (POMP)*, which encodes a protein that supports the incorporation of the β5i subunit.

All mutations were predicted to be pathogenic, and they affected proteasome assembly and maturation in an in vitro expression system. When studying activity and proteasome content in patient hematopoietic cells, Brehm et al. (16) found that the function was impaired in patients with digenic inheritance, although less severely than in patients with two *PSMB8*/β5i mutations. As expected, cells from patients with digenic CANDLE showed a defect in upregulating proteasome activities in an IFN environment compared with patients with other autoinflammatory interferonopathies. When the relevant genes were silenced in fibroblasts, additive depletion of two subunits led to more severe assembly defects and a decrease in proteolytic function, consistent with digenic inheritance. Based on a probabilistic model of proteasome assembly in which mutant subunits are competent for assembly, under a digenic conceptual schema only 6.25% of proteasomes in double-heterozygous cells would still be wild type, whereas in single-heterozygous cells, 25% of proteasomes would still be wild type. Because the protein product of *POMP* is a chaperone that is essential for proteasome maturation, mutations likely cause haploinsufficiency.

The mutations in digenic CANDLE/PRAAS patients described here affect multiple proteolytic functions of the proteasome (Figure 5), suggesting a possible role for measurement of proteasome activity as an adjunct to genetic testing in the evaluation of certain patients. It is also clear that whole-exome sequencing may be more informative than targeted genetic diagnostics in patients suspected of having CANDLE/PRAAS. The example of CANDLE/PRAAS also suggests the possibility of digenic inheritance in other unexplained autoinflammatory phenotypes, the discovery of which will be facilitated by the synergy of genomic sequencing and our emerging understanding of innate immune pathways.



Almost two decades ago, two independent consortia identified *Mediterranean fever (MEFV)*, the gene mutated in *FMF*, by positional cloning (41, 60). FMF had long been a topic of intense interest among students of both genetics and inflammation because of its high frequency in Mediterranean and Middle Eastern populations, the unexplained recurrent or even periodic nature of its inflammatory attacks, and the potentially fatal development of amyloidosis as a consequence of untreated disease. Both as a practical matter of facilitating diagnosis and as a matter of scientific enquiry, *MEFV* was a major target in the positional cloning era.

In some ways, the eventual discovery of this gene more than lived up to expectations. Genetic testing has permitted the assignment of a firm diagnosis in ambiguous cases and the recognition of a broader spectrum of disease than was hitherto appreciated, in many cases legitimizing or even introducing the possibility of life-altering treatment that had not



previously been considered. Having a molecular genetic test for FMF not only catalyzed the delineation of several distinct periodic fever syndromes that are now distinguished from FMF at the clinical, molecular, and therapeutic levels (6, 37, 39, 52, 55), but also set the stage for the eventual recognition of [autoinflammatory diseases](#) as an important nosologic category (89).

At a mechanistic level, the recognition of pyrin, the protein encoded by *MEFV*, has also been consequential (25). Its presence in [innate immune](#) cells such as neutrophils, monocytes, dendritic cells, and serosal and synovial fibroblasts suggested a major role in the regulation of inflammation. The recognition of the eponymous ~90-residue PYRIN domain at the N terminus of pyrin defined a cognate interaction motif that is present in more than 20 human proteins involved in the regulation of inflammation and apoptosis, and provided a major molecular clue for how pyrin might regulate innate immunity (86, 102). This ultimately led to the firm recognition of [IL-1 \$\beta\$](#)  as a central mediator of inflammation in FMF (9, 20, 26, 50, 74, 96, 103) and the initially surprising insight—based both on studies of *MEFV* heterozygotes (7, 12, 13, 18, 117) and on Chae et al.'s (24) seminal work on FMF knock-in mice—that disease-associated pyrin mutations lead to a gain of function through an ASC- and caspase-1-dependent pathway.

Nevertheless, a major mystery remained unsolved until recently: Does the pyrin protein serve as a sensor for some sort of microbial infection? That this might be the case was suggested both by the extraordinarily high carrier frequencies for *MEFV* that have been directly documented in certain populations, indicating the possibility of a selective advantage for the carriers (25), and by the fact that other innate immune molecules mutated in autoinflammatory disease, such as the above-mentioned [NLRP4](#) and NLRP3, serve precisely such a function as pathogen sensors. Because there are strong data that IL-1 $\beta$  plays a central role in FMF, one can put the question in a somewhat different way: Is there a pyrin [inflammasome](#), and if so, what triggers its activation?

### 5.1. Feng Shao and the Holy Grail

In 2006, Alnemri and colleagues (140) suggested the existence of a pyrin inflammasome that is responsible for IL- $\beta$  production. However, it was unclear at the time how that inflammasome would be regulated. More recently, some pieces of the puzzle that came from a completely different direction have started to fall into place.

The first evidence that pyrin might form an inflammasome in response to certain bacteria came from Wewers and colleagues (45). Human monocytes infected with *Francisella tularensis* released substantial amounts of cleaved IL-1 $\beta$ , which could be diminished by small interfering RNA against pyrin. Moreover, THP-1 cells transfected with pyrin induced IL-1 $\beta$  production in response to stimulation with the same bacterium. Similarly, a correlation was found between pyrin expression and IL-1 $\beta$  production in response to *Burkholderia cenocepacia* (44) that was dependent on expression of ASC and the bacterial type 6 secretion system.

In their studies of host-pathogen interactions, Shao and colleagues (138, 139) have recently provided an elegant molecular mechanism for the activation of the pyrin inflammasome. They discovered that pyrin is activated upon bacterial toxin-induced modification of host Rho GTPases, a known bacterial virulence mechanism that blocks host defense by preventing assembly of the cytoskeleton (138, 139). This mechanism of pathogen detection is conceptually different from other inflammasomes because the pyrin inflammasome senses bacterial virulence activity rather than a specific bacterial product. This indirect activation model is

reminiscent of the [guard mechanism](#) by which innate immunity is triggered in plants (66).

Various Rho-inactivating bacterial toxins are known, modifying the switch I region of Rho GTPases in several ways to activate the pyrin inflammasome. The TcdB toxin from *Clostridium difficile* monoglucosylates RhoA at Thr37, the C3 toxin from *Clostridium botulinum* and the pertussis toxin from *Bordetella pertussis* ADP-ribosylate RhoA at Asn41, the *Vibrio parahaemolyticus* VopS and *Histophilus somni* IbpA toxins adenylylate RhoA at Tyr34 or Thr37, and *Burkholderia cenocepacia* deamidates RhoA at Asn41 (138, 139). Because multiple modifications on multiple RhoA residues induce pyrin inflammasome activation, it is unlikely that pyrin recognizes the modifications per se; instead, it seems to be triggered by some downstream effect of the modifications.

Using bone marrow–derived macrophages from various knockout mice, Shao and colleagues (138, 139) demonstrated that caspase-1 activation, IL- $\beta$  production, and pyroptosis induced by these bacterial toxins were dependent on murine pyrin and ASC but not on NLRP3, NLRC4, or AIM2. Taken together, these data make a strong case that pyrin is an indirect sensor for certain bacterial toxins and suggest that the selective advantage for *MEFV* mutations may be related to the possibility that such mutations increase pyrin inflammasome activity and contribute to host defense.

## 5.2. The Next Frontier

The discovery of the physiologic role of pyrin is a great step forward, but several questions now emerge. Because it is unlikely that bacterial toxins interact directly with pyrin, current research attempts to elucidate the mechanism by which RhoA modification leads to the assembly of the pyrin inflammasome (99). These studies focus on the working hypothesis that RhoA modifications affect the phosphorylation status of pyrin, changing the affinity of pyrin for one of its binding partners, 14-3-3 (63, 99). This work also investigates the effect of FMF-associated mutations on pyrin inflammasome activity, and thus how such mutations may have been selected over human history and whether there are any identifiable environmental factors that might serve to trigger attacks in patients. Current studies also address the mechanism of action of colchicine, a mainstay of treatment for FMF (35, 47, 143), demonstrating how it affects the pathway connecting bacterial toxins with the pyrin inflammasome (99). Finally, this work has established a previously unrecognized molecular connection between FMF and hyperimmunoglobulinemia D with periodic fever syndrome (99, 116), consistent with the fact that the underlying biochemical defect in the latter disorder affects RhoA prenylation (51, 73, 82, 127). Thus, although FMF may be the oldest recognized autoinflammatory disease, it remains a topic of intense interest to clinicians and basic scientists alike.



## 6.1. Haploinsufficiency of A20

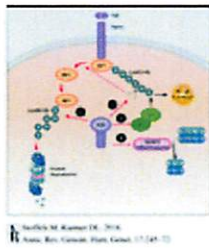
Aksentjevich and colleagues (147) recently described high-penetrance heterozygous germline mutations in the [NF- \$\kappa\$ B](#) regulatory protein tumor necrosis factor  $\alpha$ -induced protein 3 (TNFAIP3, also called A20) in six unrelated families from different ancestries who presented with early-onset systemic inflammation, oral/genital ulcers, uveitis, and arthritis/arthralgia. The phenotype resembles that of Behçet's disease, a genetically [complex disorder](#) with early adult onset (122). Whole-exome



sequencing demonstrated that all of the patients had heterozygous truncating mutations in A20. Frameshift mutations in A20 had previously been reported only in tumor tissues (81).

The A20 protein is dually involved in negatively regulating NF- $\kappa$ B signaling, both by cleaving Lys63-linked ubiquitin chains from complexes of effector molecules and by adding Lys48-linked ubiquitin chains to the dissociated proteins. Removal of Lys63-linked ubiquitin chains leads to disaggregation of signaling complexes, whereas the addition of Lys48-linked ubiquitin chains to the constituent proteins targets them for proteasomal degradation, resulting in the strong inhibition of NF- $\kappa$ B signaling (28, 57, 134). In patient cells, wild-type A20 expression was reduced and mutant A20 was not detectable. Overexpression studies showed that mutant A20 did not exert a dominant negative effect on the ability of wild-type protein to suppress TNF-induced NF- $\kappa$ B activity, nor did it interfere with the Lys63-deubiquitination function of wild-type A20. For these reasons, this new illness has been termed haploinsufficiency of A20 (HA20) (147).

The resulting increased NF- $\kappa$ B activity led to increased expression of NF- $\kappa$ B target genes in patient immune cells. Levels of proinflammatory cytokines were elevated, there was an increased polarization toward Th9 and Th17 T cell lineages, and patients had more CD14<sup>+</sup> inflammatory monocytes. In concordance with studies in mice, and independent of its role in NF- $\kappa$ B regulation, A20 haploinsufficiency led to constitutive activation of the NLRP3 inflammasome (38, 131). **Figure 6** summarizes the results from this study (147). Although this study was the first report of rare high-penetrance, germline-inactivating *TNFAIP3* mutations, common low-penetrance A20 variants have been linked to several human autoimmune diseases (80).



**Figure 6**

## 6.2. The Genetic Landscape of Behçet's Disease

The example of HA20 brings us back full circle to the quotation from Sir William Harvey at the beginning of this review: A deep analysis of the rare can inform our understanding of the common. HA20 represents an illness that is in the clinical spectrum of Behçet's disease and informs our understanding of the pathophysiology of sporadic forms of the disease, and may provide a rational basis for targeted therapies. As reviewed by Takeuchi et al. (122) and illustrated in **Figure 7**, the genetic landscape of Behçet's disease now spans the panorama from common, low-penetrance variants to rare, high-penetrance mutations, such as those seen in HA20. Other rare disorders discussed in this review (**Table 1**) may similarly shed light on their more common incarnations: [DADA2](#) and stroke or PAN, [SCAN4/NLRC4-MAS](#) and [MAS](#), [SAVI](#) and interstitial lung disease, mosaic CAPS and adult-onset Still's disease, [PRAAS](#) and idiopathic panniculitis. As illustrated by the story of the pyrin inflammasome, rare [autoinflammatory diseases](#) can provide profound insights into normal biology; as illustrated by HA20, they may also provide inroads into understanding their more common cousins.



### Figure 7

**Click to view table**

**Table 1** Autoinflammatory diseases: new tricks discussed in this review

1. New techniques in next-generation sequencing have led to a quantum jump in the number of recognized monogenic autoinflammatory diseases as well as the pathways that underlie their pathogenesis.
2. Patients with deficiency of adenosine deaminase 2 (DADA2) have mutations in the *CECR1* gene, which leads to reduced serum levels of ADA2, impaired endothelial development, and skewed macrophage differentiation.
3. STING-associated vasculopathy with onset in infancy (SAVI) mainly involves inflammation in the skin, blood vessels, and lungs. Mutations in the STING molecule lead to constitutive activation of the STING–IFN- $\beta$  pathway.



4. Macrophage activation syndrome (MAS) is a life-threatening systemic immune dysregulatory condition with uncontrolled macrophage activation. Mutations in *NLRC4* lead to overproduction of IL-1 $\beta$  and IL-18. High IL-18 serum levels remain even during anti-IL-1 treatment, suggesting an important role for IL-18 in MAS.
5. More complicated mechanisms are being unraveled, such as the digenic mode of inheritance in proteasome-associated autoinflammatory syndrome (PRAAS). Heterozygous mutations in proteins that form a complex, or compound heterozygous mutations in a single protein in that complex, can lead to disease. Digenic inheritance is more challenging to detect; hypothesis-driven analysis can help to facilitate gene discovery.
6. Late-onset cryopyrin-associated periodic syndrome (CAPS) and Schnitzler syndrome can be caused by mosaic mutations in the *NLRP3* gene in myeloid cells that drive inflammation. Mosaicism can explain the later onset of the disease and a slightly different phenotypic spectrum from patients with germline mutations.
7. The physiologic function of pyrin, the protein mutated in familial Mediterranean fever (FMF), is to activate IL-1 $\beta$  in response to bacterial toxins that modify RhoA GTPase. Because it senses physiologic changes in the cell rather than specific bacterial products, pyrin is the first documented example in mammalian innate immunity of the guard mechanism first described in plants.
8. Haploinsufficiency of A20 (HA20) is part of the Behçet's disease spectrum. Whereas most Behçet's phenotypes are thought to result from a combination of common genetic variants with low effect, HA20 is caused by rare variants with high effect. This results in insufficient function of the A20 protein, which is ordinarily responsible for inhibiting NF- $\kappa$ B and IL-1 $\beta$  signaling.



1. As genomic sequencing becomes increasingly available, data sharing and the functional analysis of sequence variants will become ever more critical.
2. Delineation of the possible roles for ADA2 in common disease, and the development of targeted therapies, are important priorities.
3. Based on the discovery of NLRC4-MAS, it will be important to investigate the role of NLRC4 and IL-18 in other, more common conditions and to develop therapies that target IL-18.

4. Development of therapies that target STING may be important in the treatment of more common diseases, such as those mediated by DNA viruses and autoimmune diseases driven by double-stranded DNA.
5. The discovery of digenic inheritance is constrained by our understanding of protein complexes and biochemical pathways. The development of hypothesis-neutral approaches to digenic and even oligogenic gene discovery would be a major advance.
6. It will be important to determine what percentage of patients with adult-onset autoinflammatory disease can be explained by mosaicism.
7. Studies to unravel the mechanism by which RhoA modifications lead to pyrin inflammasome activation are under way. How do FMF-associated pyrin mutations affect this pathway? Does the mechanism of action of colchicine relate to this pathway?
8. The rare *TNFAIP3* high-effect variants that cause HA20 have been described, but the percentage of Behçet's patients that can be explained by sporadic gene variants remains to be determined. Do common variants in *TNFAIP3* contribute to predisposition to Behçet's disease?



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*DIDA: Digenic diseases DAtabase.* <http://dida.ibsquare.be> (see also Gazzo AM, Daneels D, Cilia E, Bonduelle M, Abramowicz M, et al. 2016. DIDA: a curated and annotated digenic diseases database. *Nucleic Acids Res.* 44:D900–97)

**DIDA: A database that provides information and insight into how variants jointly lead to disease.**

*Infervers: the registry of hereditary auto-inflammatory disorders mutations.* <http://fmf.igh.cnrs.fr/ISSAID/infervers>



**Infevers: A database of all mutations found in autoinflammatory disorders.**